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Sodium Dodecyl Sulfate-Eethylenediaminetetraacetic Acid Sensitive Phenotype Associated with *ompC* Deficient *Escherichia coli* Strains is Observed Primarily in Cells Growing in Stationary Phase and Less So in Cells Growing in Log Phase

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The highly conserved, multi-component Mla inter-membrane lipid trafficking system is proposed to be involved in maintaining outer membrane lipid asymmetry. It has been shown that MlaA interacts with both the OmpC and OmpF porins. OmpC deficient strains have been shown to accumulate phospholipids in the outer membrane outer leaflet during stationary phase, indicating a role in lipid asymmetry maintenance during stationary phase. ∆*ompC* cells are known to have outer membrane permeability defects and show increased sensitivity to sodium dodecyl sulfate (SDS) and ethylenediaminetetraacetic acid (EDTA) treatment. In this study, we investigated the importance of OmpC in maintaining lipid asymmetry using an adapted minimum inhibitory concentration assay (MIC). Previous studies have tested the sensitivity of ∆*ompC* mutant strains of *Escherichia coli*. We hypothesized that complementing *ompC* using a plasmid would restore a SDS-EDTA resistant phenotype to these strains. Previous studies have shown that ∆*ompC* cells are affected in stationary phase. We looked at SDS-EDTA sensitivity of ∆*ompC* mutant JW2203 and the importance of the porin during both stationary and log-phase growth of *E. coli*. Growth phase experiments suggest that JW2203 ∆*ompC* mutants are more sensitive to the effects of SDS-EDTA during stationary phase than log phase, and this observation is consistent with previous studies. Overall, our data suggests that growth phase may impact the SDS-EDTA sensitivity of *E. coli* strain JW2203.

The outer membrane (OM) of Gram-negative bacteria acts as a barrier to toxic compounds such as detergents, bile salts, and antibiotics. A unique feature of the OM cell membrane is its asymmetric lipid distribution, with phospholipids (PLs) confined to the inner leaflet, and lipopolysaccharide (LPS) molecules accumulated on the outer leaflet. Due to the strong lateral interactions between LPS molecules and the OM's low fluidity, this asymmetry contributes to making the membrane more hydrophobic than a typical phospholipid bilayer. The combination of a highly hydrophobic lipid bilayer and the porins within it having specific size-exclusion properties make the OM a strongly selective barrier for bacterial cells (4).

It is known that the lipopolysaccharide transport (Lpt) machinery brings LPS to the OM, and provides transportation to the outer leaflet via the LptD/E OM complex (3). The process by which PLs are transported to the OM remains unclear. Despite being stabilized by divalent cations, the asymmetric configuration of the OM is energetically unfavorable and PLs have a net tendency to flip from the inner to the outer leaflet (3). Subsequently, once the OM asymmetry has been established, there are several cell mechanisms in place to prevent PL occurrence in the OM outer leaflet (3). One of the systems in place to prevent this movement of PLs is the highly conserved, multi-component Mla inter-membrane lipid trafficking system, which is proposed to be involved in maintaining OM lipid asymmetry. First proposed by Malinverni *et al.* (8), this system involves six proteins at various positions across the cell envelope: MlaA as an OM lipoprotein, MlaC as a periplasmic protein, and MlaFEDB forming an ATP-binding cassette in the inner membrane (IM). While it has been seen that the deletion of the Mla system causes PLs to accumulate in the OM outer leaflet, making cells more sensitive to ethylenediaminetetraacetic acid (EDTA) and sodium dodecyl sulfate (SDS) treatment, it is not known how the system works to maintain the asymmetry (3). It is proposed that the pathway begins with extraction of PLs from the OM mediated by MlaA (8). As an OM lipoprotein, MlaA is anchored to the inner leaflet and has no clear way to access the outer leaflet (3). MlaA knockout mutants show accumulation of phospholipids in the outer leaflet of the outer membrane (8), so there is strong support behind the idea that MlaA is involved in removing PLs from the outer leaflet, and would thus require some other component to mediate this interaction (3).

It has been shown that MlaA interacts with both OmpC and OmpF specifically, and that this interaction is sufficient to localize MlaA lacking its lipid anchor to the OM (3). Furthermore, ∆*ompC* mutants accumulate PLs in the OM outer leaflet during stationary phase, therefore OmpC may play a role in lipid asymmetry maintenance during stationary phase (3). OmpC is thought to be an additional OM component of the Mla system, and complexes with MlaA to extract PLs from the outer leaflet (3). Two models for this interaction have been proposed by Chong *et al.* (3): A. OmpC may allow PLs in the outer leaflet to flip back inwards, where MlaA would then remove them, or B. OmpC may allow MlaA to access the outer leaflet PLs directly by shuttling it to the surface. In either model, it is proposed that once PLs are removed from the OM, they are shuttled back to the IM using the other components of the Mla system. Once there, the fate of PLs is unknown. In both

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cases, it is not clear by what mechanism OmpC achieves the transport of either the PLs or MlaA.

Disruption of lipid asymmetry via accumulation of PLs in the outer leaflet renders cells more sensitive to external insults (11). In order to assess the disruption of OM permeability and lipid asymmetry in various Δ*ompC* mutants, one can test the susceptibility of cells to EDTA and SDS, external agents that can damage the cell and prevent growth by disrupting lipid stability and solubilizing the OM. Due to disruption of OM asymmetry, Δ*ompC* cells are known to have OM permeability defects and, subsequently, increased sensitivity to EDTA/SDS (3). LPS is known to bind strongly to divalent cations as a way to combat the electrostatic repulsion between neighboring LPS molecules, which typically have multiple negative charges (4). EDTA chelates divalent cations and this treatment prevents LPs from making strong lateral interactions and disrupts the barrier function (9). SDS is an ionic detergent that dissolves the plasma membrane of cells. It can incorporate into the cell membrane and solubilize lipids and proteins found there. This creates pores in the membrane which leads to cells lysis (2). A minimum inhibitory concentration (MIC) assay is a commonly used technique to determine sensitivity to antimicrobial agents. Using a broth dilution of EDTA and SDS at increasing concentrations, one can determine the concentration at which different strains are unable to survive this treatment and assess the effects of *ompC* mutations on these results.

Chong *et al.* found that Δ*ompC E. coli* cells showed PL accumulation in the OM outer leaflet. According to their study, defects in outer membrane stability in Δ*ompC* mutant strains only occur during stationary phase (OD_{600nm}: 2-4) and not during exponential cell growth (3). They hypothesized that PLs may more readily accumulate in the outer leaflet in the stationary phase than when in exponential cell growth, due to the effects of OmpC deficiency. As well they postulated that differences may arise from the change in metabolism and morphology that occur when cells are in stationary phase (3). We decided to test this hypothesis by comparing SDS/EDTA sensitivity of Δ*ompC* BW2203 cells and wild-type BW25113 cells in log and stationary phase. According to Chong *et al.*, we expected decreased growth of JW2203 cells as compared to wild-type in stationary phase.

In this study, we assessed the effect of growth phase on SDS/EDTA resistance in an Δ*ompC E. coli* mutant. We found that *ompC* knockouts resulted in decreased growth during stationary phase as compared to log phase, which supports the results of Chong *et al.* These results support the findings that the absence of the OmpC porin confers susceptibility to SDS/EDTA, and suggests that this phenotype can be observed primarily in stationary phase and less so in log phase.

MATERIALS AND METHODS

Bacterial strains. *E. coli* strains used in this study are Δ*ompC* JW2203 and wild-type BW25113. Strains were grown at 37°C in Luria Bertani broth in the presence of either 10 μg/mL kanamycin (strain JW2203) or both 10 μg/mL kanamycin and 12.5 μg/mL chloramphenicol (strain JW2203 transformed with pHSG575 or pOmpC20). Strain BW25113 was grown in the absence of antibiotic selection.

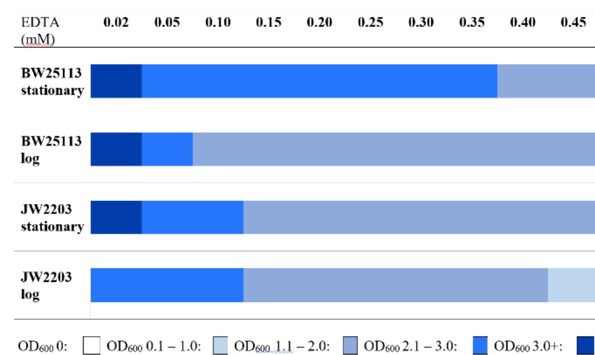
Competent cell prep and transformation. *E. coli* JW2203 cells were made competent and transformed according to the Hancock Laboratory Procedure (5). Cells were grown to log phase (0.2-0.5 OD_{550nm}) and then made competent using the calcium chloride Heat Shock Protocol. Transformation of *E. coli* JW2203 was done using pOmpC20 and pHSG575. Cells were spread plated onto LB plates containing 10 μg/mL kanamycin and 12.5 μg/mL chloramphenicol and put in the 37°C incubator for 24 hours. Plates were stored in the 4°C fridge and competent cells were stored in the -20°C freezer.

Minimum inhibitory concentration assay. MIC assays were conducted in 96 well polypropylene plates using 0.0125% SDS in each test well, with varying concentrations of EDTA at 0.02 mM, 0.05 mM, 0.1 mM, 0.15 mM, 0.2 mM, 0.25 mM, 0.3 mM, 0.35 mM, 0.4 mM, and 0.45 mM, according to the procedure done by Hartstein *et al.* (6). Each well was filled with 100uL LB containing the necessary antibiotics, 50uL of both SDS and EDTA at 4X concentrations, as well as IPTG (1mM) and glucose (0.5%) where applicable. The cells were grown to log phase (0.2-0.5 OD_{550nm}) on a 37ºC shaker. Log phase or overnight cells were seeded at a concentration of 1 x 10^6 cells/mL in the wells. Plates were incubated at 37°C overnight. Growth was assessed by determining optical density at a wavelength of 600nm using the plate reader. OD_{600} values were normalized to the negative controls on each plate, and any resulting negative values were reported as 0, for no growth.

RESULTS

Impact of growth phase on the resistance of *ompC* deficient *E. coli* strain JW2203 and wild-type *E. coli* strain BW25113 to EDTA/SDS. In order to study whether *ompC* knockouts have a detrimental effect on cell growth during the stationary-phase, as proposed by Chong *et al*. (3), we compared the optical density values of wild type and *ompC* knockout strains of *E. coli* during log- and stationary-phases of cell growth. JW2203 Δ*ompC* strain was generated as part of the Keio collection, using recombination with a kanamycin cassette (1). Hartstein *et al*. showed that this strain was sensitive to EDTA/SDS (6), and Chong *et al.* suggested that the sensitivity of Δ*ompC* mutant strains of *E. coli* to EDTA/SDS is predominantly seen in stationary phase. However, it is not clear whether the sensitivity phenotype of JW2203 observed by Hartstein *et al*. is limited to stationary phase cells or if log phase cells show the same phenotype. To address this question, we used a MIC to compare the growth of an Δ*ompC* mutant and wild type *E. coli* strain growing in stationary or log phase. The MIC conditions used by Hartstein *et al.* were employed in this experiment. We obtained seven sample repeats for the BW25113 stationary and log phase cells and six sample repeats for the JW2203 stationary and log phase cells. For each sample repeat, the controls worked as expected. Cells inoculated in the absence of EDTA/SDS were positive for growth and media without inoculum was negative for growth. According to Table 1, the wild-type control cells showed resistance in stationary phase but not in log phase. There was also no notable difference between the wild type and mutant cells in log phase. There is a distinguishable difference between the level of growth observed between the wild type cells and

TABLE 1 Comparing stationary phase and log phase BW25113 and JW2203 *E. coli* cells in the EDTA/SDS MIC assay. JW2203 Δ*ompC E. coli* grown in LB-Kan, BW25113 *E. coli* grown in LB, at 37ºC overnight.



the mutant cells during stationary phase. This data indicates that growth phase impacts SDS-EDTA resistance of JW2203, as stationary phase cells display increased susceptibility.

Rescue of SDS-EDTA phenotype in *ompC* deficient *E. coli* strain JW2203 by complementation with pOmpC20. In order to study how the *ompC* knockout leads to EDTA/SDS resistance, we attempted to complement JW2203 log phase cells with the OmpC20 plasmid, which contains the wild type *ompC* gene. We also complemented JW2203 log phase cells with pHSG575, an empty vector to account for the effects of the vector itself in the MIC. We expected that JW2203 cells containing the empty vector would have a phenotype resembling uncomplemented JW2203 cells. The MIC assay was repeated 8 times using log phase JW2203, BW25113 and complemented JW2203 with pOmpC20 and pHSG575 separately. JW2203 cells complemented with the empty vector appeared to have increased growth in comparison to the JW2203 cells. JW2203 cells complemented with pOmpC20 appeared to have increased growth compared to the wild-type BW25113. Both of these results are unexpected and the data is inconclusive at this point.

DISCUSSION

The OM of Gram-negative bacteria is composed of an asymmetric bilayer containing phospholipids located in the inner leaflet and LPS on the outer leaflet. The asymmetric lipid distribution contributes to an increased hydrophobicity of the bilayer and in conjunction with size-specific porins embedded in the bilayer, allows the OM to act as a strongly selective barrier for cells (4). The integration of LPS into the outer leaflet of the OM occurs through transport by the Lpt machinery, yet it is unknown how phospholipids are transported to the OM (3). It is proposed that the Mla inter-membrane lipid trafficking system is involved in maintaining OM asymmetry and that it interacts with both OmpC and OmpF (3). OmpC appears to play a role in maintaining asymmetry, but its mechanism is unclear.

We tested the effect of an *ompC* knockout at different growth phases to determine at what point OmpC becomes involved in maintaining lipid asymmetry. We found that there was a noticeable difference in EDTA/SDS sensitivity between wild-type and *ompC* knockout cell growth during stationary phase, but not in log phase. Since this process of maintaining lipid asymmetry is integral to the survival of *E. coli*, there are likely several systems in place to keep PLs out of the OM outer leaflet throughout the life of the cell. The Mla system, in conjunction with OmpC, appears to serve this purpose during the stationary phase of the cell cycle. Our results agree with the hypothesis put forward by Chong *et al.* which proposes that OmpC is important for EDTA/SDS resistance during stationary phase growth of *E. coli* cells.

Log phase cells grow exponentially while stationary phase cells grow linearly. Chong *et al.* demonstrated that the phenotypic differences between log and stationary phase cells is not due to Mg2+ limitation in stationary phase, but rather that it may stem from differences in the nature of OM defects in the two growth phases (3). They hypothesized that the accumulation of PLs in the outer leaflet may occur more readily when cells are in stationary phase and that these cells would require OmpC to remove PLs in order to maintain lipid asymmetry, whereas log phase cells would not (3). In response to nutrient limitations, stationary phase cells undergo metabolic and morphological changes, and demonstrate altered PL metabolism and lipid homeostasis, which could affect OM lipid asymmetry (3). These growth phase associated adjustments may account for the differences in OM defects, and requirement of OmpC involvement in the different phases of *E. coli* growth. This may explain the differences seen in EDTA/SDS sensitivity between log and stationary phase cells in this experiment.

Additionally, we attempted to complement the JW2203 strain *ompC* deficient phenotype with pOmpC20, a vector containing the wild type *ompC* gene, but results were variable. We expected that wild-type BW25113 and the pOmpC20 complemented JW2203 cells would show similar growth patterns, but the complemented cells grew much better than the wild-type cells. Also, we expected that the mutant JW2203 cells and the pHSG575 complemented JW2203 cells would show similar growth patterns, but the complemented cells grew better than both the JW2203 and BW25113 cells. These results indicate that the experiment did not work as expected, and needs to be approached differently. It is unclear how to best induce gene expression from the plasmid, although both the native *ompC* promoter and *lacZ* promoter are found on the plasmid. These promoters can be induced by glucose and IPTG respectively, so we performed MIC assays using different combinations of these inducers in the media. As our results from this

experiment were variable, it is still unclear which combination produces the most accurate results. Furthermore, we cannot be sure whether the plasmids are even being induced under these conditions, or if the OmpC protein is being expressed. Overall our results are inconclusive regarding the effect of complementation of JW2203 with pOmpC20.

FUTURE DIRECTIONS

Future work could involve looking at *ompC* gene expression from the plasmid using qPCR or RT-PCR to determine if the plasmid is actually being expressed. This assay would also be useful for determining the effect of different inducers on *E. coli* growth in the presence of EDTA/SDS.

OmpC clearly plays a role in some resistance mechanism to EDTA/SDS. It is known that it interacts with the Mla system to maintain OM lipid asymmetry, but the mechanism by which it does so remains unclear. Chong *et al*. have proposed two models that show the role of the OmpC porin in this process, and it appears that the channel of OmpC may be key to its function in this capacity. It has been seen that OmpC is able to transport unfolded protein substrates within its channel, which indicates that both hydrophilic and hydrophobic residues can move through the pore (10). It is plausible therefore, that amphipathic PLs may pass through as well and that in either model, OmpC is able to transport its substrate through its channel. It would be interesting to investigate the effects of OmpC channel mutations on its ability to participate in maintaining lipid asymmetry, and whether these mutations confer sensitivity to EDTA/SDS. In addition to the two plasmids used in this study (pOmpC20 and pHSG575), we have also obtained a plasmid used by Harstein *et al.* last year, originally constructed by Lou *et al.* (pOmpC33, Dr. J.H. Naismith, Centre for Biomolecular Sciences, University of St. Andrews), that contains three point mutations, producing amino acid substitutions within the channel of OmpC. Lou *et al.* showed that these mutations restricted the passage of antibiotics through the OM into the *E. coli* cells and conferred resistance. We have sequenced the pOmpC33 plasmid and confirmed the presence of the expected mutations (Fig. S1). We had difficulties transforming plasmid pOmpC33 into JW2203, and also had further trouble growing the transformed cells. Future workers could try to optimize growth conditions needed for the expression of pOmpC33, and subsequently, investigate the sensitivity OmpC33 expressing cells to EDTA/SDS.

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